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(54) PEPTIDES AND THEIR PREPARATION

(71) We, MERCK & CO. INC., a corporation duly organised and existing under the laws of the State of New Jersey, United States of America, of Rahway, New Jersey, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us and the method by which it is to be performed, to be particularly described in and by the following statement: —

This invention provides hemopeptides P1, P2, H and S, new synthetic peptides in which hemopeptide P1 has the peptide chain H-val-his - leu - ser - ala - glu - glu - lys - glu-ala - OH; hemopeptide P2 has the peptide chain H - val - his - leu - ser - ala - glu-glu-lys-gln-ala-OH, hemopeptide H has the peptide chain H - val - his - leu - thr - pro-glu - glu - lys - ser - ala - OH; and hemopeptide S has the peptide chain H - met - leu-thr - ala - glu - glu - lys - ala - ala - OH; their amides, esters and N-acyl derivatives, and protected derivatives thereof. The invention is concerned with processes for preparing these hemopeptides by controlled stepwise synthesis whereby individual amino acid components of the peptide chain are connected in the stated sequence by peptide linkages. The invention also provides certain intermediate polypeptides produced in the processes, viz, the protected derivatives of the pentapeptide H-glu - glu - lys - glu - ala - OH, the pentapeptide H - val - his - leu - ser - ala - OH, the pentapeptide H - glu - glu - lys - gln-ala - OH, the pentapeptide H - glu - glu - lys-ser - ala - OH, the pentapeptide H - val - his-leu - thr - pro - OH, the pentapeptide H-glu - glu - lys - ala - OH, and the tetrapeptide H - met - leu - thr -ala - OH. More particularly, the new hemopeptides are prepared either by successively introducing each of the individual amino acid components stepwise in sequence, or by synthesizing two or more segments of the peptide chain followed by coupling of such segments in the stated sequence.

Hemopeptides P1, P2, H and S are useful in inducing release and/or synthesis of hormone substances by living systems, and are particularly valuable for effecting release of growth hormone by cells of the anterior pituitary. They are conveniently administered by injection, preferably by intracarotoid injection where release and/or synthesis of growth hormone is desired.

Abbreviated designations for the amino acid components, their derivatives, and certain preferred protecting groups used in this specification are as follows:

Amino Acid	Abbreviated Designation
L-alanine	ala
L-glutamic acid	glu
L-glutamine	gln
L-histidine	his
L-leucine	leu
L-lysine	lys
L-methionine	met
L-proline	pro
L-serine	ser
L-threonine	thr
L-valine	val

Derivatives; Protecting Groups	Abbreviated Designation
N-carboxyanhydride	NCA
N-thiocarboxyanhydride	TCA
Benzyloxycarbonyl (carbobenzoxy)	Cbz
Tertiary-butoxycarbonyl	tBOC
N-hydroxysuccinimide ester	NHS
Methyl ester	OMe

In accordance with the present invention, hemopeptides P1, P2, H and S are prepared by stepwise coupling (by peptide linkages) of their respective individual amino acid components, which peptide coupling is conducted by reacting the appropriate amino acid in the sequence (as a derivative in which the carboxyl grouping is activated and any amino groups are protected) first with alanine (the amino acid at the C-terminus i.e. carboxy end of the polypeptide chain), and then subsequently with each resulting polypeptide intermediate, such stepwise method being referred to herein as sequential synthesis. When this sequential synthesis conducted in solution, it is ordinarily preferred to utilize, as the carboxyl-activated amino acid, the amino acid NCA, the amino acid TCA, the amino acid azide, or an activated ester such as the NHS ester of such amino acid. These NCA and TCA sequential synthesis procedures are more fully described in French Patent 1,497,536.

Alternatively, the hemopeptides P1, P2, H and S are prepared using solid phase sequential synthesis procedure starting from the C-terminus. In this procedure. the carboxyl end of the terminal amino acid, alanine (and of the polypeptide product in the following steps), is bound covalently to an insoluble polymeric resin support, as for example as the carboxylic ester of the resin-bonded benzyl alcohol present in hydroxymethyl-substituted polystyrene-divinylbenzene resin. In this solid phase procedure, the peptide coupling may involve direct condensation between the free carboxyl of an amino acid reactant and the amino group of the resin-bonded alanine or polypeptide. The reaction is ordinarily conducted in the presence of a coupling agent such as dicyclohexylcarbodiimide, although the amino acid reactant may be in the form of a carboxyl-activated amino acid such as the NHS ester or an amino acid azide.

Instead of sequential synthesis, hemopeptides P1, P2, H and S can also be prepared by block synthesis, in which various peptide segments of the hemopeptide chains are individually synthesized, and these are then coupled in proper sequence to form the desired polypeptide product. These peptide segments are themselves conveniently prepared by sequential synthesis in solution using NCA, TCA, azide or NHS ester procedure, or by activated NHS ester or amino acid azide or, if desired, a free carboxyl-containing amino acid reactant in conjunction with a coupling agent. The number of amino acid components in the peptide segments used in block synthesis of hemopeptides P1, P2, H and S may vary from two to eight, but peptide segments containing five amino acid components or fewer are preferably used, thus avoiding condensations involving larger peptide segments with attendant losses of these more valuable higher peptide fragments.

In carrying out these sequential or block synthesis, involving reaction between carboxyl (or activated carboxyl) of one amino acid and amino grouping of the other, it is ordinarily preferred to protect the amino groupings in the amino acid or peptide undergoing reaction at the carboxyl end of the molecule, as well as other functional groupings in both reactants reactive under the conditions of such syntheses Protecting groups must retain their protecting properties under the peptide coupling conditions, and must be selectively removable without affecting peptide linkages. Protecting groups to be removed following a particular step must also be selectively removable without affecting other protecting groups to be retained in later coupling steps.

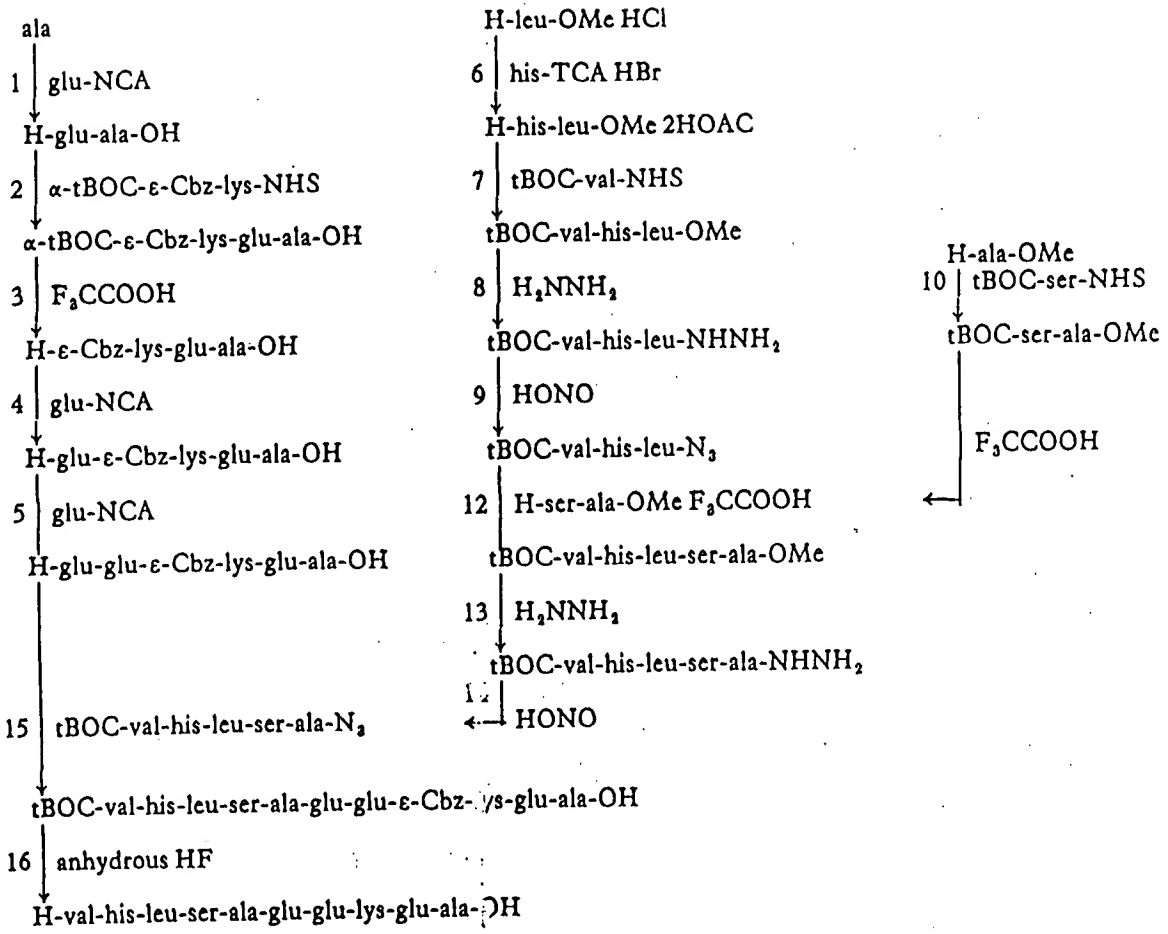
Amino-protecting groups ordinarily used include salt formation, which is particularly useful for protecting strongly-basic amino groups, acyl-type substituents such as formyl, phthalyl, trifluoroacetyl, toluenesulfonyl, dibenzylphosphoryl, nitrophenylsulfenyl, tritylsulfenyl and *o* - nitrophenoxyacetyl, urethane protecting substituents such as benzyloxycarbonyl (carbobenzoxy), *p* - methoxycarbobenzoxy, *p*-nitrocarbobenzoxy, *t* - butyloxycarbonyl, 2 - (*p*-biphenylyl) - 2 - propyloxycarbonyl and isonicotinyloxycarbonyl, and alkyl-type substituents such as triphenylmethyl, trialkylsilyl and trimethyl - silyl. It is preferred to use tert-butoxycarbonyl (tBOC) for protecting the α-amino group in the amino acids (or peptides) undergoing reaction at the carboxyl end of the molecule, since the tBOC protecting group is readily removed following the reaction and prior to the subsequent step (when the α-amino group itself undergoes reaction) by relatively mild action of acids (e.g. trifluoroacetic acid for 5—10 minutes). The mild acid treatment does not affect groupings such as carbobenzoxy (Cbz) and isonicotinyloxycarbonyl, which are used to protect other amino groups such as the ϵ-amino group of lysine, and are removable by vigorous action of a strong acid cleaving agent (e.g. hydrogen bromide in glacial acetic acid or anhydrous hydrogen fluoride in the presence of anisole for approximately one hour).

Carboxyl-protecting groups ordinarily used include amides, salt-formation, ester substituents such as the methyl and ethyl esters (which

the hydrazide, to the azide is desired), the benzyl ester, and particularly the resin-bonded benzyl ester, used in solid-phase synthesis (which reacts directly with hydrazine to cleave the peptide from the resin and form the peptide hydrazide), *p* - nitrobenzyl - ester, and *t* - butyl ester. Hydroxy groupings are ordinarily not protected in the synthesis of the hemopeptides where the coupling reactions are conducted in solution, although groups such as tetrahydropyranyl, benzyl, the trifluoroacetyl and *t*-butyl, may be used for such protection if desired. It is usually preferred, however, to use these O-protecting substituents, and particularly the O-benzyl and O - *t* - butyl groups, preparing hemopeptide P1, or the serine- or threonine-containing segments in the chain by solid-phase synthesis. The imidazole nitrogen of histidine may also be protected, if desired, preferably using an N-hydrocarbon (or substituted-hydrocarbon) substituent such as N-benzyl or N-(2,4 - dinitrophenyl).

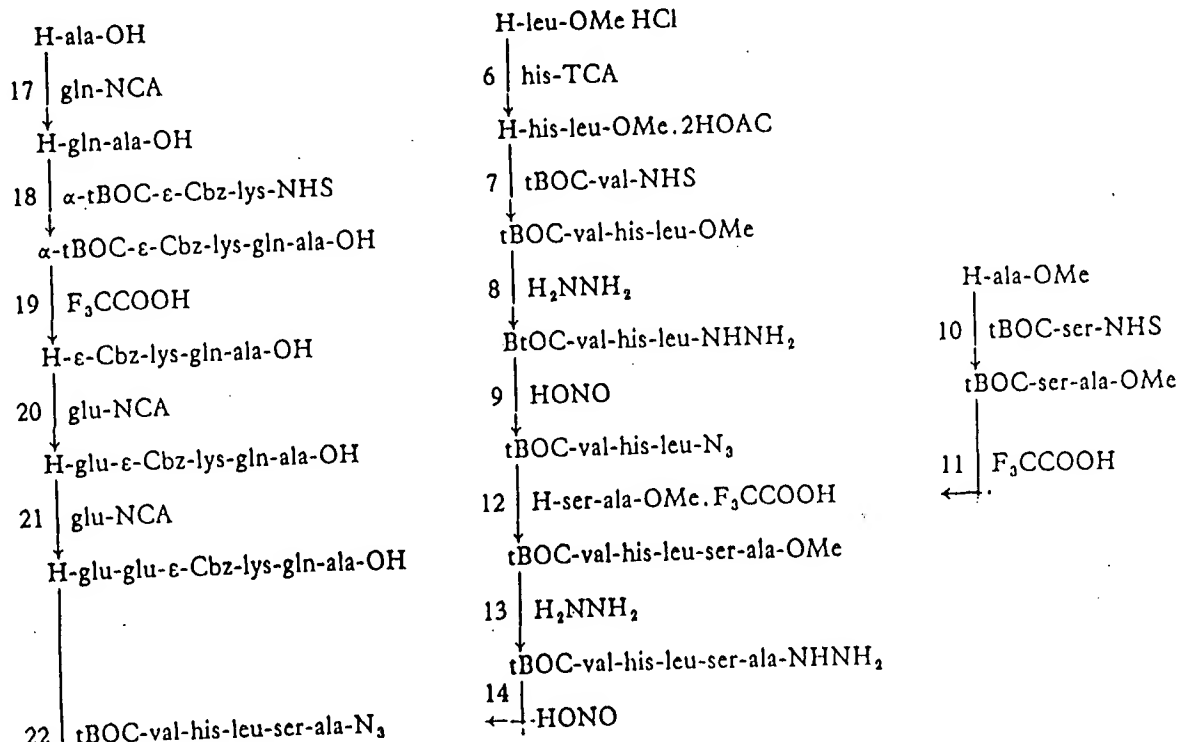
The selection of protecting groups is in part dictated by particular coupling conditions, in part by the amino acid and peptide components involved in the reaction. Guides for selecting particular protecting groups to be employed herein are set forth in detail in the aforesaid French Patent 1,497,536.

The preferred overall procedure for preparation of hemopeptide P1 is set forth diagrammatically in the following reaction scheme 1.



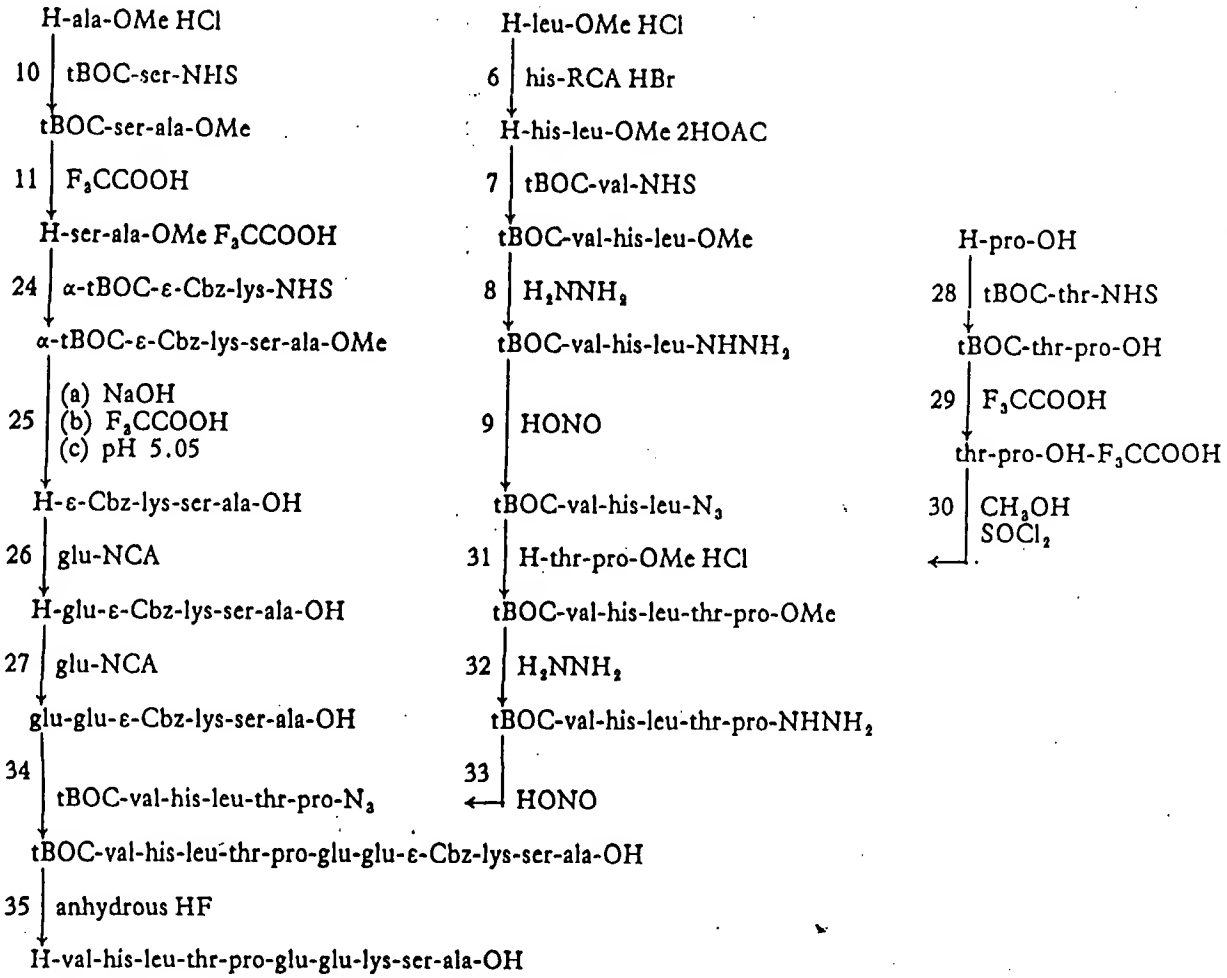
Note that numbers next to arrows refer to working Examples.

The preferred overall procedure for preparation of hemopeptide P2 is set forth diagrammatically in the following reaction scheme 2:



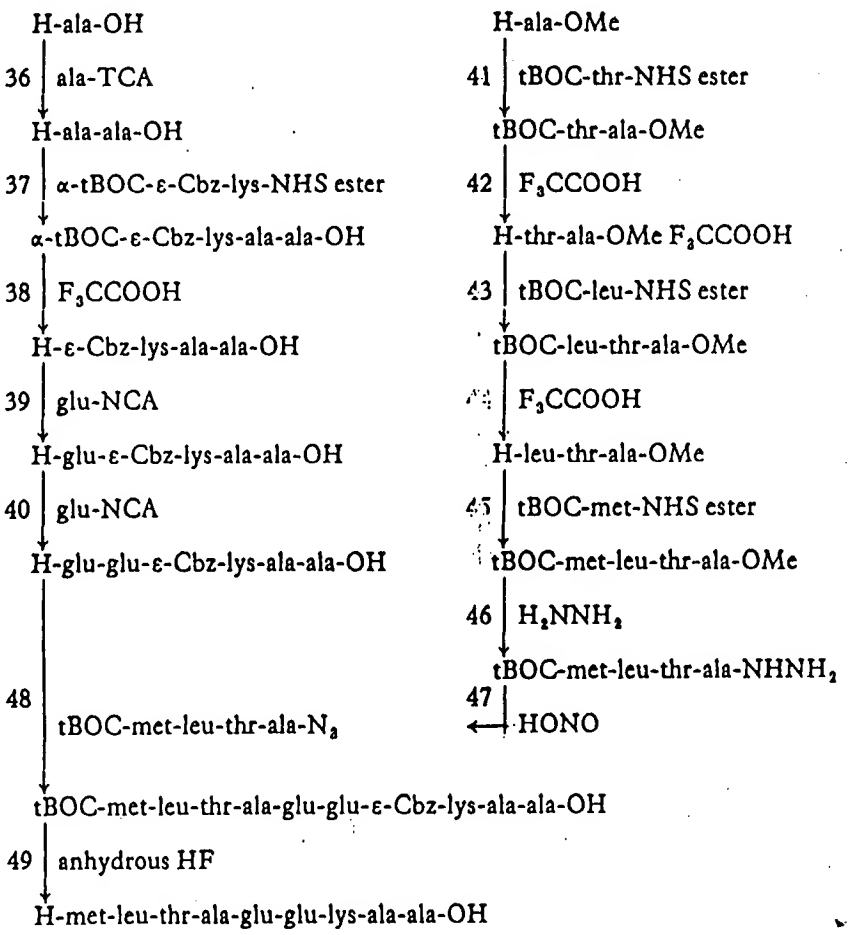
Note that numbers next to arrows refe · to working Examples.

The preferred overall procedure for preparation of hemopeptide H is set forth diagrammatically in the following reaction scheme:



Note that numbers next to arrows refer to working Examples.

The preferred overall procedure for preparation of hemopeptide S is set forth diagrammatically in the following reaction scheme 4.:



Note that numbers next to arrows refer to working Examples.

This preferred overall procedure involves the combination of sequential and block syntheses, in which certain peptide segments of the decapeptide chain are initially formed by the stepwise method, either by sequential synthesis in solution or by solid phase sequential synthesis and these segments are then coupled in proper sequence. In this procedure, the tBOC substituent is used to protect α-amino groupings, the Cbz substituent is used to protect the ϵ-amino group of lysine, and the methyl ester substituent is used to protect the carboxy groups of alanine, leucine, leucine - threonyl-alanine, histidyl - leucine, seryl - alanine, threonyl - alanine and threonyl - proline; the methyl ester also serves the further purpose of providing the intermediate compounds for preparing, via the hydrazide, tBOC - val - his-leu azide, tBOC - val - his - leu - ser - ala azide and tBOC - val - his - leu - thr - pro azide. Instead of this preferred method, however, the present invention also contemplates the various permutations of alternative routes, and use of other protecting groupings fulfilling criteria hereinabove discussed, such alternative routes likewise involving sequential synthesis in solution, sequential synthesis in solid phase, and combinations of sequential and block synthesis procedures.

As reference to reaction scheme 1 will show, the preferred overall procedure for preparing hemopeptide P1 specifically involves sequential synthesis in solution of (a) the protected pentapeptide segment, H - glu - glu - ϵ - Cbz-lys - glu - ala - OH, and (b) the carboxyl-activated, protected pentapeptide segment, tBOC - val - his - leu - ser - ala azide. The former pentapeptide is preferably prepared by reacting alanine with glu NCA, which reaction is conducted by vigorously agitating the reactants together in aqueous solution at pH 10.2, under which conditions the reaction is ordinarily complete in one to two minutes. The alkaline solution is acidified thereby decomposing the intermediate carbamate to form an aqueous solution of H - glu - ala - OH. An approximately equal volume of ethanol is added, and the aqueous ethanolic solution of H - glu - ala - OH is reacted with the NHS ester of α - tBOC - ϵ - Cbz - lysine, preferably while maintaining the pH at about 8.0, to form α - tBOC - ϵ - Cbz - lys - glu - ala-OH. This protected tripeptide is preferably reacted with trifluoroacetic acid, thereby removing the tBOC protecting group, to form H - ϵ - Cbz - lys - glu - ala - OH, which protected tripeptide is precipitated from aqueous solution by adjusting the pH to 4.1.

This H - ϵ - Cbz - lys - glu - ala - OH is reacted with the N - carboxyanhydride of glutamic acid (glu-NCA), which reaction is preferably conducted by bringing the reactants together in aqueous solution with vigorous reaction is ordinarily complete in one to two minutes. The alkaline reaction solution is acidified, thereby decomposing the intermediate carbamate to form a solution of H - glu-ϵ - Cbz - lys - glu - ala - OH which after adjusting the pH to 10.2, is then reacted with vigorous agitation with additional glu-NCA. The reaction solution is acidified, thereby decomposing the intermediate carbamate, and the pH of the aqueous solution is adjusted to 3.5, thereby precipitating the protected pentapeptide, H-glu-glu-ϵ-Cbz-lys-glu-ala-OH.

The terminal dipeptide segment of the other pentapeptide, in the form of its methyl ester trifluoroacetate, H - ser - ala - OMe F_3CCOOH, is prepared by first reacting alanine methyl ester with the NHS ester of N-tBOC - serine, preferably in dimethylformamide solution under alkaline conditions (preferably pH 8.0), thereby forming tBOC - ser-ala - OMe; this dipeptide is treated with trifluoroacetic acid thereby forming H - ser-ala-OMe trifluoroacetate.

The tripeptide in the hemopeptide P1 sequence still to be synthesized, namely val-his-leu, is prepared by reacting leucine methyl ester with the N - thiocarboxy - anhydride of histidine (which is added as his - TCA HBr), preferably in aqueous solution with vigorous agitation at pH 9.5, under which conditions the reaction is ordinarily complete in about 20 minutes. The alkaline reaction solution is acidified thereby decomposing intermediate thiocarbamate, and the acidified reaction solution is adjusted to pH 5.0, filtered to remove impurities, and evaporated *in vacuo* to give the dipeptide H-his-leu-OH in the form of its methyl ester; the crude material so obtained is purified by chromatography on silica gel using n-butanol : acetic acid : water as eluant to give H - his - leu - OMe diacetate. This H - his - leu - OMe diacetate is reacted with the NHS ester of N - tBOC - valine preferably in dimethylformamide solution under mildly alkaline conditions (preferably ph 8.0) to form the tripeptide, tBOC - val-his - leu - OMe, which is reacted with hydrazine, and the resulting hydrazide treated with nitrous acid, thereby forming tBOC - val-his - leu azide.

The tBOC - val - his - leu azide is reacted with H - ser - ala - OMe trifluoroacetate preferably in dimethylformamide solution under mildly alkaline conditions (preferably pH 8.0) to form the corresponding protected pentapeptide, tBOC - val - his - leu - ser-ala - OMe, which is, in turn, reacted with hydrazine, and the hydrazine treated with nitrous acid, thereby forming tBOC - val - his-leu - ser - ala azide.

The two protected pentapeptides, glu - glu-ϵ - Cbz - lys - glu - ala and tBOC - val - his-leu - ser - ala azide are preferably reacted, in dimethylformamide solution under mildly

by forming the protected decapeptide, tBOC-val - his - leu - ser - ala - glu - glu - ε-Cbz - lys - glu - ala - OH.

The tBOC - val - his - leu - ser - ala - glu - glu - ε - Cbz - lys - glu - ala - OH, or other protected derivative of hemopeptide P1, is then subjected to the vigorous action of a strong acid cleaving agent, for example an anhydrous hydrohalic acid *per se*, or in solution in substantially anhydrous non-hydroxylic, substantially non-basic, organic solvent for said hydrohalic acid, as for example anhydrous hydrogen fluoride, hydrogen bromide in glacial acetic acid, and hydrogen chloride in ethyl acetate, thereby removing the protecting groups to form H-val-his-leu-ser-ala-glu-glu-lys - glu - ala - OH, the unsubstituted hemopeptide P1. It is generally advantageous to use in this strong acid-cleaving reaction a carbonium ion scavenger such as anisole, veratrole, dimethylsulfide and methionine, which serves to trap the liberated protecting substituent, following cleavage, in a non-reactive form.

As reference to reaction scheme 2 will show, the preferred overall procedure for preparing hemopeptide P2 specifically involves sequential synthesis in solution of (a) the protected pentapeptide segment, H - glu - glu-ε - Cbz - lys - gln - ala - OH, and (b) the carboxyl-activated, protected pentapeptide segment tBOC - val - his - leu - ser - ala azide. The former pentapeptide is prepared by reacting alanine with gln NCA, which reaction is preferably conducted by vigorously agitating the reactant together in aqueous solution at pH 10.2, under which conditions the reaction is ordinarily complete in one to two minutes. The alkaline solution is acidified thereby decomposing the intermediate carbamate to form an aqueous solution of H - gln - ala - OH. An approximately equal volume of ethanol is added, and the aqueous ethanolic solution of H - gln - ala - OH is reacted with the NHS ester of α - tBOC - ε - Cbz - lysine, preferably while maintaining the pH at about 8.0, to form α - tBOC - ε - Cbz - lys - gln - ala - OH. This protected tripeptide is reacted with trifluoroacetic acid, thereby removing the tBOC protecting group, to form H - ε - Cbz - lys-gln - ala - OH, which protected tripeptide is precipitated from aqueous solution by adjusting the pH to 5.2.

The H-ε-Cbz-lys-gln-ala-OH is reacted with the N - carboxyanhydride of glutamic acid (glu-NCA), which reaction is preferably conducted by bringing the reactants together in aqueous solution with vigorous agitation at pH 10.2, under which conditions reaction is ordinarily complete in one to two minutes. The alkaline reaction solution is acidified, thereby decomposing the intermediate carbomate to form a solution of H - glu-ε - Cbz - lys - gln - ala - OH which, after vigorous agitation with additional glu-NCA. The reaction solution is acidified, thereby decomposing the intermediate carbamate, and the pH of the aqueous solution is adjusted to 3.7, thereby precipitating the protected pentapeptide H - glu - glu - ε - Cbz - lys - gln-ala - OH.

The other pentapeptide in protected form, namely the tBOC - val - his - leu - ser - ala-OMe, is prepared as hereinabove described in connection with the synthesis of hemopeptide P1, and the latter is, in turn, reacted with hydrazine, and the hydrazine treated with nitrous acid, thereby forming tBOC - val-his - leu - ser - ala azide.

The two protected pentapeptides, H - glu-glu - ε - Cbz - lys - gln - ala - OH and tBOC - val - his - leu - ser - ala azide are reacted in dimethylformamide solution under mildly alkaline conditions (preferably pH 8.0), thereby forming the protected decapeptide, tBOC - val - his - leu - ser - ala - glu - glu-ε - Cbz - lys - gln - ala - OH.

The tBOC - val - his - leur - ser - ala - glu-glu - ε - Cbz - lys - gln - ala - OH, or other protected derivative of hemopeptide P2, is then subjected to the vigorous action of a strong acid cleaving agent as for example anhydrous hydrohalic acid *per se*, or in solution in substantially anhydrous nonhydroxylic, substantially non-basic, organic solvent for said hydrohalic acid, as for example anhydrous hydrogen fluoride, hydrogen bromide in glacial acetic acid, or hydrogen chloride in ethyl acetate, thereby removing the protecting groups to form H - val - his - leu - ser - ala - glu-glu - lys - gln - ala - OH, the unsubstituted hemopeptide P2. It is generally advantageous to utilize in this strong acid cleaving reaction a carbonium ion scavenger such as anisole, veratrole, dimethyl sulfide, or methionine, which serves to trap the liberated protecting substituent, following cleavage, in a non-reactive form.

As reference to reaction scheme 3 will show, the preferred overall procedure for preparing hemopeptide H specifically involves sequential synthesis in solution of (a) the protected pentapeptide segment, H - glu - glu - ε - Cbz-lys - ser - ala - OH, and (b) the carboxyl-activated, protected pentapeptide segment, tBOC - val - his - leu - thr - pro azide. The former pentapeptide is prepared by reacting alanine methyl ester with the NHS ester of N - tBOC - serine preferably in dimethylformamide under mildly alkaline conditions (preferably pH 8.0). The resulting dipeptide, tBOC-ser - ala - OMe is subjected to selective treatment with acid, preferably trifluoroacetic acid, thereby removing the tBOC protecting group and forming the free α - amine, H - ser - ala-OMe. The H - ser - ala - OMe is reacted, preferably in dimethylformamide, with the NHS ester of α - tBOC - ε - Cbz - lysine to

OMe. The protected tripeptide is reacted first with aqueous alkali thereby hydrolysing the methyl ester grouping; and then with trifluoroacetic acid, thereby removing the tBOC protecting group, to form H - ε - Cbz - lys - ser-ala - OH, which protected tripeptide is precipitated from aqueous solution by adjusting the pH to 5.05.

The H - ε - Cbz - lys - ser - ala - OH is reacted with the N - carboxyanhydride of glutamic acid (glu-NCA), which reaction is preferably conducted by bringing the reactants together in aqueous solution with vigorous agitation at pH 10.1, under which conditions reaction is ordinarily complete in one to two minutes. The alkaline reaction solution is acidified, thereby decomposing the intermediate carbamate to form a solution of H - glu-ε - Cbz - lys - ser - ala - OH which, after adjusting the pH to 10.1, is then reacted with vigorous agitation with additional glu - NCA. The reaction solution is acidified, thereby decomposing the intermediate carbamate, and the pH of the aqueous solution is adjusted to 3.6, thereby precipitating the protected pentapeptide H - glu - glu - ε - Cbz - lys - ser-ala - OH.

The terminal dipeptide segment of the other pentapeptide, in the form of its methyl ester hydrochloride H - thr - pro - OMe HCl, is prepared by first reacting proline with the NHS ester of N - tBOC - threonine, preferably in dimethylformamide solution under alkaline conditions (preferably pH 10.0), thereby forming tBOC - thr - pro - OH; this dipeptide is treated with trifluoroacetic acid thereby forming H - thr - pro - OH trifluoroacetate. This thr-pro trifluoroacetate is then reacted with a methylating agent, for example methanol in the presence of thionyl chloride catalyst, thereby forming the methyl ester of H - thr - pro - OH which is recovered from the reaction mixture in the form of its hydrochloride.

The tripeptide in the hemopeptide H sequence still to be synthesized, namely H-val - his - leu - OH, is prepared as hereinbefore described in connection with hemopeptide P1.

The tBOC - val - his - leu azide, obtained as hereinbefore described, is reacted with H - thr - pro - OMe, preferably in dimethylformamide solution under mildly alkaline conditions (preferably pH 8.0), to form the corresponding protected pentapeptide, tBOC-val - his - leu - thr - pro - OMe, which is, in turn, reacted with hydrazine, and the hydrazide treated with nitrous acid, thereby forming tBOC - val - his - leu - thr - pro azide.

The two protected pentapeptides H - glu-glu - ε - Cbz - lys - ser - ala - OH and tBOC - val - his - leu - thr - pro azide, are reacted in dimethylformamide solution under mildly alkaline conditions (preferably pH 8.0), tBOC - val - his - leu - thr - pro - glu-glu - ε - Cbz - lys - ser - ala - OH.

The tBOC - val - his - leu - thr - pro - glu-glu - ε - Cbz - lys - ser - ala - OH or other protected derivative of hemopeptide H, is then subjected to the vigorous action of a strong acid cleaving agent as for example anhydrous hydrohalic acid *per se*, or in solution in anhydrous non-hydroxylic, non-basic, organic solvent for said hydrohalic acid, as for example anhydrous hydrogen fluoride, hydrogen bromide in glacial acetic acid, and hydrogen chloride in ethyl acetate, thereby removing the protecting groups to form H - val - his - leu-thr - pro - glu - glu - lys - ser - ala - OH, the unsubstituted hemopeptide H.

As reference to reaction scheme 4 will show, the preferred overall procedure for preparing hemopeptide S specifically involves sequential synthesis in solution of (a) the protected pentapeptide segment, H - glu - glu - ε - Cbz - lys-ala - ala - OH and (b) the carboxyl-activated protected tetrapeptide segment, tBOC - met-leu - thr - ala azide. The stated pentapeptide is prepared by reacting alanine with ala TCA, which reaction is preferably conducted by vigorously agitating the reactants together in aqueous solution at pH 9.5, under which conditions the reaction is ordinarily complete in about ten minutes. The alkaline solution is acidified thereby decomposing the intermediate carbamate to form an aqueous solution of H - ala - ala - OH. An approximately equal volume of ethanol is added, and the aqueous ethanolic solution of H - ala - ala - OH is reacted with the NHS ester of α - tBOC - ε - Cbz - lysine, preferably while maintaining the pH at about 8.0, to form α - tBOC - ε - Cbz - lys - ala - ala-OH. This protected tripeptide is reacted with trifluoroacetic acid, thereby removing the tBOC protecting group to form H - ε - Cbz-lys - ala - ala - OH, which protected tripeptide is precipitated from aqueous solution by adjusting the pH to 4.1.

This H - ε - Cbz - lys - ala - ala - OH is reacted with the N - carboxy - anhydride of glutamic acid (glu-NCA), which reaction is preferably conducted by bringing the reactants together in aqueous solution with vigorous agitation at pH 10.0, under which conditions reaction is ordinarily complete in one to two minutes. The alkaline reaction solution is acidified thereby decomposing the intermediate carbamate to form a solution of H - glu - ε-Cbz - lys - ala - ala - OH which, after adjusting the pH to 10.0, is then reacted with vigorous agitation with additional glu-NCA. The reaction solution is acidified thereby decomposing the intermediate carbamate, and the pH of the aqueous solution is adjusted to 4.1, the water is evaporated *in vacuo*, and the residual material is purified by chromatography to give the protected pentapeptide

The terminal dipeptide segment of the tetrapeptide residue, in the form of its methyl ester trifluoroacetate, H - thr - ala - OMe F_3CCOOH is prepared by first reacting alanine methyl ester with the NHS ester of tBOC-threonine, preferably in dimethylformamide solution under alkaline conditions (preferably pH 8.0), thereby forming tBOC - thr - ala-OMe; this dipeptide is treated with trifluoroacetic acid thereby forming thr - ala-OMe trifluoroacetate. This dipeptide is then reacted, preferably in dimethylformamide solution, with the NHS ester of tBOC - leu-OH to form tBOC - leu - thr - ala - OMe; this protected tripeptide is treated with trifluoroacetic acid thereby removing the tBOC protecting group to form H - leu - thr - ala-OMe. The latter is then reacted with the NHS ester of tBOC - met, preferably in dimethylformamide solution, to form the protected tetrapeptide, t - BOC - met - leu - thr - ala-OMe, which is, in turn, reacted with hydrazine, and the hydrazide treated with nitrous acid, thereby forming tBOC - met - leu - thr-ala azide.

The protected pentapeptide, H - glu - glu-ε - Cbz - lys - ala - ala - OH is reacted with this tBOC - met - leu - thr - ala azide in dimethylformamide solution under mildly alkaline conditions (preferably pH 8.0), thereby forming the protected nonapeptide, tBOC-met - leu - thr - ala - glu - glu - ε - Cbz - lys-ala - ala - OH.

The tBOC - met - leu - thr - ala - glu - glu-ε - Cbz - lys - ala - ala - OH, or other protected derivative of hemopeptide S, is then subjected to the vigorous action of a strong acid cleaving agent as for example anhydrous hydrohalic acid *per se*, or in solution in a substantially anhydrous nonhydroxylic substantially non-basic organic solvent for the hydrohalic acid, as for example anhydrous hydrogen fluoride, hydrogen bromide in glacial acetic acid, or hydrogen chloride in ethyl acetate, thereby removing the protecting groups to form H - met - leu - thr - ala - glu - glu - lys-ala - ala - OH, the unsubstituted hemopeptide S. It is generally advantageous to utilize in this strong acid cleaving reaction a carbonium ion scavenger such as anisole, veratrole, dimethyl sulfide, or methionine, which serves to trap the liberated protecting substituent, following cleavage, in a non-reactive form.

While it is preferred, in the practice of this invention, to use the foregoing protected tetrapeptide, pentapeptide, nonapeptide and decapeptide derivatives, other amino-protecting groups such as those outlined herein above may be used; in addition the carboxy group of alanine, the imidazole nitrogen of histidine, and the hydroxy groups of threonine and serine may also be protected as hereinabove disclosed. Thus, the novel peptides of the present invention include hemopeptides P1, P2, H and protected derivatives of the intermediate H-met - leu - thr - ala - OH and pentapeptides H - val - his - leu - ser - ala - OH, H - glu-glu - lys - glu - ala - OH, H - glu - glu-lys - gln - ala - OH, H - val - his - leu-thr - pro - OH, H - glu - glu - lys - ser-ala - OH and H - glu - glu - lys - ala - ala-OH, in which derivatives the α-amino groups of valine and methionine, and the ε-amino group of lysine are protected with amino protecting substituents characterized as being removable without substantial cleavage of peptide bonds; and in which the hydroxyl group of threonine, and/or the hydroxyl group of serine are optionally protected by protecting groups similarly characterized as being removable without substantial cleavage of peptide bonds.

Hemopeptides P1, P2, H and S, their amides such as H - val - his - leu - ser - ala-glu - glu - lys - glu - ala - NH_2, H - val - his-leu - ser - ala - glu - glu - lys - gln - ala-NH_2, H - val - his - leu - thr - pro - glu-glu - lys - ser - ala - NH_2, and H - met - leu-thr - ala - glu - glu - lys - ala - ala - NH_2, their esters such as H - val - his - leu - ser-ala-glu - glu - lys - glu - ala - OH acetate; H-val-his - leu - ser - ala - glu - glu lys - glu - ala-OH phosphate, H-val-his-his-leu-ser-ala-glu-glu-lys-gln-ala-OH acetate, H-val-his-leu-ser - ala - glu - glu - lys - gln - ala - OH phosphate, H - val - his - leu - thr - pro - glu-glu - lys - ser - ala-OH acetate, H - val - his-leu - thr - pro- glu - glu - lys - ser - ala - OH phosphate, H - met - leu - thr - ala - glu-glu - lys - ala - ala - OH acetate, H - met-leu - thr - ala - glu - glu - lys - ala - ala-OH phosphate, their N-acyl derivatives such as N - formyl - val - his - leu - ser - ala - glu-glu - lys - glu - ala - OH, N - acetyl - val-his - leu - ser - ala - glu - glu-lys-glu-ala-OH, N - formyl - val - his - leu - ser - ala - glu-glu - lys - gln - ala - OH, N - acetyl - val-his - leu - ser - ala - glu - glu - lys - gln - ala-OH, N - formyl - val - his - leu - thr - pro-glu - glu - lys - ser -ala - OH, N - acetyl-val - his - leu - thr - pro - glu - glu - lys-ser - ala - OH, and N - formyl - met - leu-thr - ala - glu - glu - lys - ala - ala - HO, as well as protected derivatives of the hemopeptides, such as tBOC - val - his - leu - ser-ala - glu - glu - ε - Cbz - lys - glu - ala - OH, tBOC - val - his - leu - ser - ala - glu - glu-ε - Cbz - lys - gln - ala - OH, tBOC - val-his - leu - thr - pro- glu - glu - ε - Cbz - lys-ser - ala - OH, tBOC - met - leu - thr - ala-glu - glu - ε - Cbz - lys - ala - ala - OH, are useful in inducing release of hormone substances by living systems of the vertebrates, and are particularly valuable for effecting release and synthesis of growth hormones by cells of the anterior pituitary of mammals; the hemopeptides P1, P2, H and S, and substituted derivatives thereof, are of value in promoting rapid and/or maximal growth of [illegible] and are of remedial value in certain

types of dwarfism. The hemopeptides and their substituted derivatives are conveniently administered by injection, or by absorption through mucous membranes (e.g. by sublingual, intra-nasal administration); oral administration may also be employed using derivatives resistant to gastric digestion. In order to obtain a direct effect on the anterior pituitary, intra-carotid injection of the hemopeptides, and their substituted derivatives, is ordinarily utilized at a dosage level of 0.05—0.5 micrograms per kg. of body weight per day to obtain growth hormone release, and up to about 0.1 milligrams per kg. of body weight for an individual dose where synthesis of growth hormone is desired.

The following Examples illustrate methods of carrying out the present invention.

Example 1.

About 1.78 g. of ala is dissolved in 200 ml. of 1M aqueous potassium borate buffer* solution (pH=10.2), the solution is cooled to about 0°C., and about 3.63 g. of glu NCA is added to the solution over a period of one minute, during which time the mixture is vigorously agitated (preferably using a Waring blender) while maintaining the temperature at 0°C. and the pH is 10.2 by the dropwise addition of 50% aqueous potassium hydroxide. The reaction is allowed to proceed, while continuing agitation and maintaining temperature at 0°C. and pH at 10.2, until base consumption ceases (about 1 minute); sufficient concentrated sulfuric acid is added to bring pH to 3.0; and nitrogen is bubbled through the acidified reaction mixture for about 15 minutes, thereby sweeping carbon dioxide from the resulting solution of H - glu - ala - OH.

Example 2.

To the aqueous solution of H - glu - ala-OH, prepared as described in Example 1, is added 200 ml. of ethanol, and the pH is adjusted to 8.0 by the addition of 50% aqueous potassium hydroxide solution. About 9.54 g. NHS ester of α - tBOC - ϵ - Cbz-lys - OH is added with stirring to this glu-ala - OH solution, while maintaining the temperature at about 25°C. and the pH at 8.0 by the dropwise addition of 50% aqueous sodium hydroxide solution. When base consumption ceases, the reaction solution is filtered, the ethanol is evaporated therefrom *in vacuo*, and the aqueous reaction solution is extracted with 300 ml. of ethyl acetate, thereby extracting unreacted NHS ester present in said solution. The pH of the aqueous reaction solution is then adjusted to 3.7 by the addition of concentrated sulfuric acid, and the acidified solution is extracted with three 300-ml. portions of ethyl acetate; these latter ethyl acetate extracts are combined and dried over anhydrous sodium sulfate, and the ethyl acetate evaporated therefrom *in vacuo* to form a heavy oil. Thin-layer chromatography on silica gel, using *n*-butanol-pyridine - acetic - acid - water (30:20:6:24) as eluant, shows the product to consist mainly of α - tBOC - ϵ - Cbz - lys - glu - ala-OH, with a trace (less than 1%) of a faster-moving impurity.

Example 3.

This α - tBOC - ϵ - Cbz - lys - glu - ala-OH oil is dissolved in about 50 ml. of methylene chloride, the solution is cooled to 15°C., about 60 ml. of trifluoroacetic acid is added, and the temperature of the mixture is allowed to rise to about 22°C. over a five minute period. The reaction solution is cooled to 0°C., and ether is added, while maintaining the temperature at 0°C., thereby precipitating H - ϵ - Cbz - lys - glu - ala - OH trifluoroacetate. The precipitated material is recovered by filtration, dissolved in water, the pH of the solution is adjusted to 4.1 by the addition of 2.5N aqueous sodium hydroxide solution, and the crystalline material which separates is recovered by filtration and dried to give about 4.5 g. of substantially pure H - ϵ - Cbz - lys-glu - ala - OH.

Example 4.

4.22 g. of the H - ϵ - Cbz - lys - glu - ala-OH is dissolved in 90 ml. of 1M aqueous potassium borate buffer solution (pH=10.2), the solution is cooled to about 0°C., and 1.60 g. of glu-NCA is added to the solution over a period of about 0.5 minutes, during which time the mixture is vigorously agitated while maintaining the temperature at 0°C. and the pH at 10.2 by the dropwise addition of 50% aqueous potassium hydroxide solution. The reaction is allowed to proceed, while continuing agitation and maintaining temperature at 0°C. and pH at 10.2, until base consumption ceases; sufficient concentrated sulfuric acid is added to bring pH to 2.5; and nitrogen is bubbled through the acidified reaction mixture for about 30 minutes, thereby sweeping carbon dioxide from the resulting solution of H - glu - ϵ - Cbz - lys - glu - ala - OH.

Example 5.

The solution of H - glu - ϵ - Cbz - lys-glu - ala - OH, prepared as described in Example 4, is adjusted to pH of 10.2 at 0°C. by the addition of 50% aqueous potassium hydroxide solution, and about 1.678 g. of glu-NCA is added to the solution over a period of about 0.5 minutes, while vigorously agitat-

*This buffer is conveniently prepared as follows: one mole of boric acid is slurried in 500 ml. of water, and solid potassium hydroxide merely sufficient to dissolve the boric acid is added; additional potassium hydroxide is then added to bring the pH to 10.2, the solution is diluted to 990 ml., the pH is again adjusted to 10.2, and the solution diluted to a

ture at 0°C. and the pH at 10.2 by the addition of 50% aqueous potassium hydroxide solution. The reaction is allowed to proceed, while continuing agitation and maintaining temperature at 0°C. and pH at 10.2, until base consumption ceases. The reaction solution is filtered; sufficient concentrated sulfuric acid is added to the filtered solution to bring the pH to 3.5; and the precipitated material is recovered by filtration, washed with water, and dried in vacuo to give about 4.9 g. of H - glu - glu - ϵ l Cbz - lys - glu - ala - OH, which may contain up to about 15% of tetrapeptide and 3% hexapeptide impurities. This material is subjected to free flowing electrophoresis at pH 7.0 in 0.143M 2,6 - lutidine-acetic acid buffer, to give about 2.32 g of substantially pure H - glu - glu - ϵ - Cbz-lys - glu - ala - OH.

Example 6.

About 4.34 g. of H - leu - OMe hydrochloride is dissolved in about 240 ml. of 1M aqueous potassium borate buffer solution (pH=9.5), the solution is cooled to about 0°C., and about 10.01 g. of his-TCA hydrobromide is added to the solution over a period of 10 minutes, during which time the mixture is vigorously agitated while maintaining the temperature at 0°C. and the pH at 9.5 by the dropwise addition of 50% aqueous potassium hydroxide solution. The reaction is allowed to proceed, while continuing agitation and maintaining temperature at 0°C. and pH at 9.5, until base consumption ceases (about 10 minutes); sufficient concentrated sulfuric acid is added to bring pH to 5.0; the acidified reaction solution is filtered, and the filtered solution is evaporated to dryness *in vacuo*. The residual material, which contains both his and H-leu-OMe, is subjected to chromatography on silica gel using *n*-butanol-acetic acid-water (10:2.3:6) as eluant, to give about 7.0 g. of substantially pure H - his - leu - OMe diacetate.

Example 7.

About 2.4 g. of H - his - leu - OMe diacetate is dissolved in 140 ml. of dimethylformamide, about 2.23 g. of NHS ester of tBOC-val is added with stirring at 25°C., and the pH of the solution is adjusted to 8.0 by the addition of triethylamine. The resulting solution is allowed to stand for a period of about 20 hours, with occasional adjustment of the pH to 8.0 by addition of triethylamine. The reaction solution is evaporated to dryness *in vacuo*, and residual material is dissolved in 400 ml. of water. The aqueous solution is extracted with three 400-ml. portions of chloroform. The combined organic extracts are dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*, and the residual oil is triturated with ether to give a crystalline material which is recovered by fil-

Example 8.

To about 2.8 g. of tBOC-val-his-leu-OMe is added 20 ml. of 1:1 mixture of anhydrous hydrazine and methanol. The resulting mixture is stirred for about 3 minutes at room temperature (solution is ordinarily complete in about 1 minute, and precipitate forms after about 2 minutes), and the reaction mixture is then evaporated *in vacuo* at a temperature of about 35°C. About 10 ml of ethanol is added to the residual material, and the resulting mixture is evaporated *in vacuo*; about 10 ml. of dimethylformamide is then added and the resulting mixture is evaporated *in vacuo*. The residual solid is dried *in vacuo* at room temperature for a period of about 15 hours, and crystallized from methanol to give about 2.2 g. of tBOC - val - his - leu hydrazide.

Example 9.

1.0 g of tBOC - val - his - leu hydrazide prepared as described in Example 8, is suspended in 30 ml. of freshly degassed dimethylformamide, and the suspension is cooled to a temperature of −40°C. and maintained under a dry nitrogen atmosphere to exclude moisture. To the cold suspension is added, with stirring, a solution of 6.24 ml. of 2N hydrogen chloride in tetrahydrofuran followed by 0.3 ml. of isoamyl nitrite. The resulting mixture is maintained under a dry nitrogen atmosphere at a temperature of −15°C. to −20°C. for a period of about 1 hour, at the end of which time the hydrazide has completely reacted to form tBOC - val - his - leu azide, as may be demonstrated by thin-layer chromatography on silica gel G using the solvent system chloroform-ethanol-water (5:5:1).

Example 10.

About 5.56 g. of H - ala - OMe hydrochloride and about 12.08 g. of the NHS ester of tBOC-ser are dissolved in 400 ml. of freshly degassed dimethylformamide. The resulting solution is adjusted to pH 8.0 by the addition of diisopropylethylamine, and stirred for a period of about four hours, while maintaining the temperature at about 25°C. and the pH at 8.0 by the addition of diisopropylethylamine. The reaction mixture is evaporated *in vacuo*, the residual oil is dissolved in methylene chloride, and the methylene chloride solution is washed twice with 0.2 N aqueous sulfuric acid solution saturated with sodium sulfate, once with saturated aqueous sodium chloride solution, twice with saturated aqueous sodium bicarbonate solution, and finally twice with saturated aqueous sodium chloride solution. The washed methylene chloride solution is then dried over anhydrous sodium sulfate, evaporated *in vacuo*, and the residual oil is crystallised from ethyl acetate - hexane to

Example 11.

About 6.0 g. of this tBOC - ser - ala-OMe is dissolved (at a temperature of about 0°C.) in the minimum quantity of trifluoroacetic acid, the solution is stirred at a temperature of about 25°C. for a period of about 45 minutes, and the solution is then added dropwise with vigorous stirring to a large volume (about 100 ml.) of ether. The material which precipitates is recovered by filtration, washed with ether, and dried *in vacuo* to give about 4.97 g. of H - ser - ala - OMe trifluoroacetate.

Example 12.

The reaction solution containing tBOC-val - his - leu azide, prepared as described in Example 9, is cooled to a temperature of −40°C., and to this is added a solution of 630 mg. of H - ser - ala - OMe trifluoroacetate in 10 ml. of degassed dimethylformamide. The pH of the resulting solution is adjusted to 8.0 by the addition of diisopropylethylamine, and the mixture is maintained at a temperature between about −20°C. and −15°C. (with periodic adjustment of the pH to 8.0 by addition of diisopropylethylamine) for a period of about 20 hours, at the end of which time the reaction to form the pentapeptide is substantially complete, as may be shown by thin layer chromatography on silica gel G using the solvent system ethyl - acetate-pyridine - acetic - acid - water (10:5:1:3). The reaction solution is evaporated *in vacuo;* the residual material is triturated with ethyl acetate; and the resulting solid material is washed three times with ethyl acetate, and crystallized from ethanol - ethyl - acetate to give about 0.8 g. of substantially pure tBOC-val - his - leu - ser - ala - OMe.

Example 13.

To about 790 mg. of tBOC - val - his-leu - ser - ala - OMe is added 9 ml. of an anhydrous 1:1 mixture of hydrazine and methanol. The resulting mixture is stirred for about 3 minutes at room temperature, at the end of which time solution is substantially complete. The resulting solution is evaporated *in vacuo* at a temperature of about 35°C., about 10 ml. of ethanol is added to the residual material, and the resulting solution is evaporated *in vacuo*. The residual material is dissolved in a minimum quantity of chloroform - methanol - water (60:40:10), and the solution is passed through a dry column of 50 g. of silica gel H, thereby removing traces of unreacted hydrazine (as shown by thin layer chromatography on silica gel G) and the solvent is evaporated *in vacuo* to give about 550 mg. of tBOC - val- his - leu - ser - ala hydrazide.

Example 14.

528 mg. of tBOC - val - his - leu - ser - ala hydrazide, prepared as described in Example 13, is dissolved in 100 ml. of freshly degassed dimethylformamide; the solution, which is maintained under a dry nitrogen atmosphere, is cooled to about −40°C., and about 9.6 ml. of 2N anhydrous hydrogen chloride in tetrahydrofuran is added with stirring. About 0.12 ml. of isoamyl nitrite is then added, and the resulting mixture is maintained at a temperature between about −20°C. and −15°C. for a period of about one hour, at the end of which time the hydrazide has completely reacted to form tBOC - val - his - leu - ser - ala azide, as may be demonstrated by thin-layer chromatography, utilizing the solvent system chloroform-methanol-water (60:40:10).

Example 15.

To the solution of tBOC - val - his - leu-ser - ala azide in dimethylformamide, prepared as described in Example 14, is added about 612 mg. of H - glu - glu - ε - Cbz - lys-glu - ala - OH, and the mixture is stirred at about −20°C. until the azide goes into solution. The temperature of the solution is then adjusted to −40°C., the pH is adjusted to 8 by the addition of diisopropylethylamine, and the solution is maintained at a temperature between about −20°C. and −15°C., with periodic adjustment of pH to 8.0 by addition of diisopropylethylamine, for a period of about 20 hours, at the end of which time the reaction to form the decapeptide is substantially complete, as may be shown by thin-layer chromatography on silica gel G using the solvent system butanol - pyridine - acetic acid-water (30:20:6:24). The reaction mixture, which contains a gelatinous precipitate, is evaporated *in vacuo;* the residual material is triturated and washed with water; and the water-washed material is dissolved in 50% aqueous acetic acid, and the solution passed through a superfine gel filtration column (thereby separating a small amount of impurity) to give about 474 mg. of tBOC - val-his - leu - ser - ala - glu - glu - ε - Cbz-lys - glu - ala - OH.

Example 16.

40 mg. of tBOC - val - his - leu - ser - ala-glu - glu - ε - Cbz - lys - glu - ala-OH is dried *in vacuo* over phosphorus pentoxide for a period of about 15 hours, thereby removing traces of water, and the resulting dry material is placed in a polyethylene tube containing about 0.3 ml. of anisole. The mixture is cooled to a temperature of about −35°C., one ml. of anhydrous hydrogen fluoride is condensed in the tube, and the resulting mixture is stirred at a temperature of about 0°C. for a period of about 45 minutes. At the end of this reaction period, a stream of dry nitrogen is passed through the mixture (still at 0°C.), thereby removing excess hydrogen fluoride. The residual material is held *in vacuo* at a temperature of about 25°C. for

a period of about 20 minutes, dissolved in aqueous acetic acid, and the aqueous acetic acid solution is freeze-dried to give about 41 mg. of amorphous product which is crystallized from water-ethanol to give substantially pure H - val - his - leu - ser - ala - glu-glu - lys - glu - ala - OH.

Example 17.

1.78 g. of ala is dissolved in 200 ml. of 1M aqueous potassium borate buffer solution (pH=10.2) (see Example 1), the solution is cooled to about 0°C., and 3.63 g. of gln NCA is added to the solution over a period of one minute, during which time the mixture is vigorously agitated (preferably using a Waring blender) while maintaining the temperature at 0°C. and the pH at 10.2 by the dropwise addition of 50% aqueous potassium hydroxide. The reaction is allowed to proceed, while continuing agitation and maintaining temperature at 0°C. and pH at 10.2, until base consumption ceases (about 1 minute); sufficient concentrated sulfuric acid is added to bring pH to 3.0; and nitrogen is bubbled through the acidified reaction mixture for about 15 minutes, thereby sweeping carbon dioxide from the resulting solution of H - gln-ala - OH.

Example 18.

To the aqueous solution of H - gln - ala-OH, prepared as described in Example 17, is added 200 ml. of ethanol, and the pH is adjusted to 8.0 by the addition of 50% aqueous potassium hydroxide solution. About 9.54 g. NHS ester of α - tBOC - ε - Cbz - lys is added with stirring to this H - gl[illegible] OH solution, while the temperature is maintained at about 25°C. and the pH at 8.0 by the dropwise addition of 50% aqueous sodium hydroxide solution. When base consumption ceases, the reaction solution is filtered, the ethanol is evaporated therefrom *in vacuo*, and the aqueous reaction solution is extracted with 300 ml. of ethyl acetate, thereby extracting unreacted NHS ester present in said solution. The pH of the aqueous reaction solution is then adjusted to 3.7 by the addition of concentrated sulfuric acid, and the acidified solution is extracted with three 300-ml. portions of ethyl acetate; these latter ethyl-acetate extracts are combined, dried over anhydrous sodium sulfate, and the ethyl acetate evaporated therefrom *in vacuo* to form a heavy oil. Thin layer chromatography on silica gel, using *n* - butanol - pyridine - acetic-acid - water (30:20:6:24) as eluant, shows the product to consist mainly of α - tBOC-ε - Cbz - lys - gln - ala - OH, with a trace (less than 1%) of a faster-moving impurity.

Example 19.

This α - tBOC - ε - Cbz - lys - gln - ala-[illegible] about 60 ml. of trifluoroacetic acid is added, and the temperature of the mixture is allowed to rise to about 22°C. over a five minute period. The reaction solution is cooled to 0°C., and ether is added, while maintaining the temperature at 0°C., thereby precipitating H - ε - Cbz - lys - gln - ala - OH trifluoroacetate. The precipitated material is recovered by filtration, dissolved in water, the pH of the solution is adjusted to 5.2 by the addition of 2.5N aqueous sodium hydroxide solution, and the crystalline material which separates is recovered by filtration and dried to give about 4.5 g. of substantially pure H - ε-Cbz - lys - gln - ala - OH.

Example 20.

4.22 g. of the H - ε - Cbz - lys - gln - ala-OH is dissolved in 90 ml. of 1M aqueous potassium borate buffer solution (pH=10.2), the solution is cooled to about 0°C., and 1.60 g. of glu-NCA is added to the solution over a period of about 0.5 minutes, during which time the mixture is vigorously agitated while maintaining the temperature at 0°C. and the pH at 10.2 by the dropwise addition of 50% aqueous potassium hydroxide solution. The reaction is allowed to proceed, while continuing agitation and maintaining temperature at 0°C. and pH at 10.2, until base consumption ceases; sufficient concentrated sulfuric acid is added to bring pH to 2.5; and nitrogen is bubbled through the acidified reaction mixture for about 30 minutes, thereby sweeping carbon dioxide from the resulting solution of H-glu-ε-Cbz-lys-gln-ala-OH.

Example 21.

The solution of H - glu - ε - Cbz - lys-gln - ala - OH, prepared as described in Example 20, is adjusted to pH of 10.2 at 0°C. by the addition of 50% aqueous potassium hydroxide solution, and about 1.678 g. of glue-NCA is added to the solution over a period of about 0.5 minutes, while the mixture is vigorously agitated and the temperature maintained at 0°C. and the pH at 10.2 by the addition of 50% aqueous potassium hydroxide solution. The reaction is allowed to proceed, while continuing agitation and maintaining temperature at 0°C. and pH at 10.2, until base consumption ceases. The reaction solution is filtered; sufficient concentrated sulfuric acid is added to the filtered solution to bring pH to 3.7; and the precipitated material is recovered by filtration, washed with water, and dried *in vacuo* to give about 4.9 g. of H-glu-glu-ε-Cbz-lys-glu-ala - OH, which may contain up to about 15% of tetrapeptide and 3% hexapeptide impurities. This material is subjected to free-flowing electrophoresis at pH 7.0 in 0.143M 2,6 - lutidine - acetic acid buffer, to give about 2.32 g. of substantially pure H - glu-

Example 22.

To the solution of tBOC - val - his - leu-ser - ala azide in dimethylformamide, prepared as described in Example 14, is added about 612 mg. of H - glu - glu - ε - Cbz - lys - gln-ala - OH, and the mixture is stirred at about −20°C. until the latter goes into solution. The temperature of the solution is then adjusted to −40°C., the pH is adjusted to 8 by the addition of diisopropylethylamine, and the solution is maintained at a temperature between about −20°C. and −15°C., with periodic adjustment of pH to 8.0 by addition of diisopropylethylamine, for a period of about 20 hours, at the end of which time the reaction to form the decapeptide is substantially complete, as may be shown by thin layer chromatography on silica gel G using the solvent system butanol - pyridine - acetic acid-water (30:20:6:24). The reaction mixture, which contains a gelatinous precipitate, is evaporated in vacuo; the residual material is triturated and washed with water; and the water-washed material is dissolved in 50% aqueous acetic acid, and the solution passed through a superfine gel filtration column (thereby separating a small amount of impurity) to give about 474 mg. of tBOC - val-his - leu - ser - ala - glu - glu - ε - Cbz - lys-gln - ala - OH.

Example 23.

About 40 mg. of tBOC - val - his - leu-ser - ala - glu - glu - ε - Cbz - lys - gln - ala-OH is dried *in vacuo* over phosphorus pentoxide for a period of about 15 hours, thereby removing traces of water, and the resulting dry material is placed in a polyethylene tube containing about 0.3 ml. of anisole. The mixture is cooled to a temperature of about −35°C., one ml. of anhydrous hydrogen fluoride is condensed in the tube, and the resulting mixture is stirred at a temperature of about 0°C. for a period of about 45 minutes. At the end of this reaction period, a stream of dry nitrogen is passed through the mixture (still at 0°C.), thereby removing excess hydrogen fluoride. The residual material is held *in vacuo* at a temperature of about 25°C. for a period of about 20 minutes, dissolved in aqueous acetic acid, and the aqueous acetic acid solution is freeze-dried to give about 40 mg. of amorphous product which is crystallized from water-ethanol to give substantially pure H - val - his - leu - ser - ala-glu - glu - lys - gln - ala - OH.

Example 24.

About 2.43 g. of H - ser - ala - OMe trifluoroacetate and about 3.82 g. of the NHS ester of α - tBOC - ε - Cbz - lys are dissolved in 85 ml. of freshly degassed dimethylformamide. The resulting solution is four hours while maintaining the temperature at about 25°C. and the pH at 8.0 by the addition of triethylamine. The reaction mixture is filtered and evaporated *in vacuo*, the residual syrup is dissolved in 200 ml. chloroform, and the chloroform solution is washed with 0.1 N aqueous sulfuric acid solution, then with saturated aqueous sodium bicarbonate solution, and finally with water. The washed chloroform solution is then dried over anhydrous sodium sulfate, evaporated *in vacuo*, and the residual oil is crystallized from ethyl acetate-ether to give about 3.15 g. of crystalline α - tBOC - ε - Cbz - lys - sera - ala-OMe.

Example 25.

A solution of about 3.15 g. of α - tBOC-ε - Cbz - lys - ser - ala - OMe in 20 ml. dioxane is added to 200 ml. of water while holding the pH at 11.5 by the addition of 1N aqueous sodium hydroxide solution. The resulting solution is stirred at a temperature of about 25°C. for a period of three hours while maintaining the pH at 11.5. The dioxane is evaporated from the reaction solution *in vacuo*, water is added to the residual solution to bring the volume to approximately 200 ml., and the aqueous solution is washed with three 200 ml.-portions of ethyl acetate. The aqueous solution is then adjusted to pH 2.5 by the addition of sulfuric acid, and the acidified aqueous solution is extracted with three 200 ml.-portions of ethyl acetate. The combined ethyl acetate extracts are evaporated *in vacuo* to give, as a residual oil, α - tBOC - ε - Cbz - lys - ser - ala - OH.

This residual oil is dissolved in 15 ml. of methylene chloride; the methylene chloride solution is cooled to about 5°C.; about 15 ml. of trifluoroacetic acid is added to the cold solution; and the resulting solution is allowed to warm to about 25°C. over a 5 minute period. The reaction solution is cooled to 0°C., about 200 ml. ether is added thereto with stirring, and the material which precipitates is recovered by filtration, washed with ether, and dried to give H - ε - Cbz - lys - ser-ala - OH trifluoroacetate.

This H - ε - Cbz - lys - ser - ala - OH trifluoroacetate is dissolved in about 25 ml. of water, the pH is adjusted to 5.05 by the addition of dilute aqueous sodium hydroxide solution, and the crystalline material which separates is recovered by filtration and dried to give about 1.4 g. of H - ε - Cbz - lys - ser-ala - OH.

Example 26.

0.88 g. of the H - ε - Cbz - lys - ser - ala-OH is dissolved in 20 ml. of 1M aqueous potassium borate buffer solution (pH=10.1), the solution is cooled to about 0°C., and about 0.36 g. of glu-NCA is added to the solution over a period of about 0.5 minutes,

while the temperature is maintained at 0°C. and the pH at 10.1 by the dropwise addition of 50% aqueous potassium hydroxide solution. The reaction is allowed to proceed, while continuing agitation and maintaining temperature at 0°C. and pH at 10.1, until base consumption ceases (approximately 1 minute); sufficient concentrated sulfuric acid is added to bring pH to 2.5, and nitrogen is bubbled through the acidified reaction mixture for about 15 minutes, thereby sweeping carbon dioxide from the resulting solution of H - glu-ε - Cbz - lys - ser - ala - OH.

Example 27.

The solution of H - glu - ε - Cbz - lys-glu - ala - OH, prepared as described in Example 26 is cooled to 0°C., the pH is adjusted to 10.1 by the addition of 50% aqueous potassium hydroxide solution, and about 0.38 g. of glu-NCA is added to the solution over a period of about 0.5 minutes, while the mixture is vigorously agitated and the temperature maintained at 0°C. and the pH at 10.1 by the addition of 50% aqueous potassium hydroxide solution. The reaction is allowed to proceed, with continuing agitation and maintenance of the temperature at 0°C. and the pH at 10.1, until base consumption ceases (approximately 1 minute). The pH of the reaction solution is adjusted to 8.5, and the solution is filtered thereby removing a small amount of precipitated material; sufficient concentrated sulfuric acid is added to the filtered solution to bring the pH to 3.6; and the material which now precipitates is recovered by filtration, washed with water, and dried *in vacuo* to give about 1.3 g. of H - glu - glu - ε - Cbz - lys - ser - ala - OH, which may contain some tetrapeptide and hexapeptide impurities. This material is subjected to free-flowing electrophoresis at pH 7.0 in 0.143M, 2,6 - lutidine - acetic acid buffer, to give about 0.2 g. of substantially pure H - glu - glu - ε - Cbz - lys - ser - ala-OH.

Example 28.

10.3 g. of pro is added to a solution of about 28.5 g. of the NHS ester of tBOC-thr in 900 ml. of freshly degassed dimethylformamide; the resulting suspension is adjusted to pH 10.0 by the addition of triethylamine; and the suspension is stirred for a period of about 60 hours, while maintaining the temperature at about 25°C. and pH at 10.0. The reaction mixture is evaporated *in vacuo*, the residual material is dissolved in 900 ml. water, the solution is filtered thereby removing a small amount of impurities, and the pH of the filtered solution is adjusted to 2.5 by addition of 6N aqueous hydrochloric acid solution. The aqueous solution is extracted evaporated *in vacuo* to give about 26 g. of amorphous tBOC - thr - pro - OH.

Example 29.

25 g. of this tBOC - thr - pro - OH is dissolved (at a temperature of about 0°C.) in approximately the minimum quantity of trifluoroacetic acid to effect solution; the solution is stirred at a temperature of about 25°C. for a period of about 7 minutes, and to the reaction solution is slowly added with stirring about 400 ml. of ether. The material which precipitates is recovered by filtration, washed with ether, and dried *in vacuo* to give about 14 g. of H - thr - pro - OH trifluoracetate.

Example 30.

To 200 ml. of cold methanol (temperature of about −10°C.) is added, dropwise with stirring, about 10 ml. of thionyl chloride. The solution is stirred at −10°C. for about 15 minutes, 10 g. of H - thr - pro - OH trifluoroacetate is added thereto, and the resulting solution is stirred at a temperature of 25°C. for a period of about three hours. The reaction mixture is filtered, the filtered solution is evaporated to dryness, the residual material is triturated with ether, and the solid amorphous material is recovered by filtration and dried to give about 7 g. of H - thr - pro - OMe hydrochloride.

Example 31.

The reaction solution containing tBOC-val - his - leu azide, prepared as described in Example 9, is cooled to a temperature of −40°C., and to this is added a solution of 555 mg of H - thr - pro - OMe hydrochloride in 10 ml. of degassed dimethylformamide. The pH of the resulting solution is adjusted to 8.0 by the addition of diisopropylethylamine, and the mixture is maintained at a temperature between about −20°C. and −15°C. (with periodic adjustment of the pH to 8.0 by addition of diisopropylethylamine) for a period of about 20 hours, at the end of which time the reaction to form the pentapeptide is substantially complete as may be shown by thin layer chromatography on silica gel G using the solvent system ethyl - acetate-pyridine - acetic - acid - water (10:5:1:3). The reaction solution is evaporated *in vacuo*, the residual material is dissolved in water, and the solution is purified by passage through Sephadex gel G-10 to give about 0.8 g. of substantially pure tBOC - val - his - leu - thr-pro - OMe.

Example 32.

To 790 mg. of tBOC - val - his - leu - thr-pro - OMe is added 9 ml. of an anhydrous 1:1 mixture of hydrazine and methanol. The resulting mixture is stirred for about 3 minutes at room temperature, at the end of which time solution is substantially complete. The

ethanol is added to the residual material, and the resulting solution is evaporated *in vacuo*. The residual material is dissolved in a minimum quantity of chloroform-methanol-water (60:40:10), and the solution is passed through a dry column of 50 g. of silica gel H, thereby removing traces of unreacted hydrazine (as shown by thin layer chromatography on silica gel G) and the solvent is evaporated *in vacuo* to give about 480 mg. of tBOC - val - his-leu - thr - pro hydrazide.

Example 33.

528 mg. of tBOC - val - his - leu - thr - pro hydrazide, prepared as described in Example 32, is dissolved in 100 ml. of freshly degassed dimethylformamide; the solution, which is maintained under a dry nitrogen atmosphere, is cooled to about −40°C., and 9.6 ml. of 2N anhydrous hydrogen chloride in tetrahydrofuran is added with stirring. 0.11 ml. of isoamyl nitrite is then added, and the resulting mixture is maintained at a temperature between about −20°C. and −15°C. for a period of about one hour, at the end of which time the hydrazide has completely reacted to form tBOC - val - his - leu - thr - pro azide, as may be demonstrated by thin-layer chromatography, utilizing the solvent system chloroform-methanol-water (60:40:10).

Example 34.

To the solution of tBOC - val - his - leu-thr - pro azide in dimethylformamide, prepared as described in Example 33, is added about 515 mg. of H - glu - glu - ε - Cbz-lys - ser - ala - OH, and the mixture is stirred at about −20°C. until the latter goes into solution. The temperature of the solution is then adjusted to −40°C., the pH is adjusted to 8 by the addition of diisopropylethylamine, and the solution is maintained at a temperature between about −20°C. and −15°C., with periodic adjustment of pH to 8.0 by addition of diisopropylethylamine, for a period of about 20 hours, at the end of which time the reaction to form the decapeptide is substantially complete, as may be shown by thin layer chromatography on silica gel G using the solvent system ethyl - acetate-pyridine - acetic - acid - water (10:5:1:3). The reaction mixture, which contains a gelatinous precipitate, is evaporated *in vacuo*, the residual material is dissolved in 50% aqueous acetic acid, and the solution passed through a G-25 fine gel filtration column (thereby separating a small amount of impurity) to give about 400 mg. of tBOC - val-his 1 leu - pro - glu - glu - ε - Cbz - lys-ser - ala - OH.

Example 35.

40 mg. of tBOC - val - his - leu - thr-pro - glu - glu - ε - Cbz - lys - ser - ala - OH is dried *in vacuo* over phosphorus pentoxide for a period of about 15 hours, thereby removing traces of water, and the resulting dry material is placed in a polyethylene tube containing about 0.3 ml. of anisol. The mixture is cooled to a temperature of about −35°C., one ml. of anhydrous hydrogen fluoride is condensed in the tube, and the resulting mixture is stirred at a temperature of about 0°C. for a period of about 45 minutes. At the end of this reaction period, a stream of dry nitrogen is passed through the mixture (still at 0°C.), thereby removing excess hydrogen fluoride. The residual material is held *in vacuo* at a temperature of about 25°C. for a period of about 20 minutes, dissolved in aqueous acetic acid, and the aqueous acetic acid solution is freeze-dried to give about 41 mg. of amorphous product which is crystallized from water-ethanol to give substantially pure H - val - his - leu - thr - pro - glu - glu-lys - ser - ala - OH.

Example 36.

1.33 g. of ala is dissolved in 200 ml. of 0.1M aqueous potassium borate buffer solution (pH=9.5) (see Example 1), the solution is cooled to about 0°C., and about 2.0 g. of ala TCA is added to the solution over a period of five minutes, during which time the mixture is stirred while the temperature is maintained at 0°C. and the pH at 9.5 by the dropwise addition of 25% aqueous sodium hydroxide. The reaction is allowed to proceed with continued stirring and maintenance of the temperature at 0°C. and pH at 9.5, until base consumption ceases (about 5 minutes); sufficient concentrated sulfuric acid is added to bring the pH to 3.0; and nitrogen is bubbled through the acidified reaction mixture for about 45 minutes, thereby sweeping carbon dioxide from the resulting solution of H-ala-ala-OH.

Example 37.

To the aqueous solution of H-ala-ala-OH, prepared as described in Example 36, is added 200 ml. of ethanol, and the pH is adjusted to 8.0 by the addition of 25% aqueous sodium hydroxide solution. About 7.15 g. NHS ester of α - tBOC - ε - Cbz - lys is added with stirring to this ala - ala solution, while the temperature is maintained at about 25°C. and pH at 8.0 by the dropwise addition of 25% aqueous sodium hydroxide solution. When base consumption ceases, the reaction solution is filtered, the ethanol is evaporated therefrom *in vacuo*, and the aqueous reaction solution is extracted with 100 ml. of ethyl acetate, thereby extracting unreacted NHS ester present in said solution. The pH of the aqueous reaction solution is then adjusted to 3.7 by the addition of concentrated sulfuric acid, and the acidified solution is extracted with three 100-ml. portions of ethyl acetate; these latter ethyl-acetate extracts are combined

the ethyl acetate is evaporated therefrom *in vacuo* to form a heavy oil. Thin-layer chromatography on silica gel, using *n* - butanol-pyridine - acetic - acid - water (30:20:6:24) as eluant, shows the product to consist mainly of α - tBOC - ε - Cbz - lys - ala - ala - OH with a trace (less than 1%) of a faster-moving impurity.

Example 38.

This α - tBOC - ε - Cbz - lys - ala - ala-OH oil is dissolved in about 30 ml. of methylene chloride, the solution is cooled to 10°C., about 36 ml. of trifluoroacetic acid is added, and the temperature of the mixture is allowed to rise to about 24°C. over a five minute period. The reaction solution is cooled to 0°C., and ether is added, while maintaining the temperature at 0°C., thereby precipitating H - ε - Cbz - lys - ala - ala - OH trifluoroacetate. The precipitated material is recovered by filtration, dissolved in water, the pH of the solution is adjusted to 4.1 by the addition of 2.5N aqueous sodium hydroxide solution, and the crystalline material which separates is recovered by filtration and dried to give about 2.0 g. of substantially pure H - ε - Cbz-lys - ala - ala - OH. About 0.7 g. of material is obtained upon evaporation of the mother liquors.

Example 39.

1.26 g. of the H - ε - Cbz - lys - ala - ala-OH is dissolved in 30 ml. of 1M aqueous potassium borate buffer solution (pH=10.0), the solution is cooled to about 0°C., and 0.544 g. of glu-NCA is added to the solution over a period of about 0.5 minutes, during which time the mixture is vigorously agitated while the temperature is maintained at 0°C. and the pH at 10.0 by the dropwise addition of 50% aqueous potassium hydroxide solution. The reaction is allowed to proceed, with continued agitation and maintenance of the temperature at 0°C. and the pH at 10.0, until base consumption ceases; sufficient concentrated sulfuric acid is added to bring pH to 2.5; and nitrogen is bubbled through the acidified reaction mixture for about 30 minutes, thereby sweeping carbon dioxide from the resulting solution of H - glu - ε - Cbz - lys-ala-ala-OH.

Example 40.

The solution of H - glu - ε - Cbz - lys-ala - ala - OH, prepared as described in Example 39, is adjusted to pH of 10.0 at 0°C. by the addition of 50% aqueous potassium hydroxide solution, and 0.570 g. of glu-NCA is added to the solution over a period of about 0.5 minutes, while the mixture is vigorously agitated and its temperature maintained at 0°C. and its pH at 10.0 by the addition of 50% aqueous potassium hydroxide solution. The reaction is allowed to proceed, with continued agitation and maintenance of solution is filtered; sufficient concentrated sulfuric acid is added to the filtered solution to bring the pH to 4.1. The water is evaporated *in vacuo*, and the residual solid material is dissolved in 25 ml. of 50% aqueous acetic acid solution. This aqueous acetic acid solution is then subjected to gel permeation chromatography, thereby removing salt and impurities to give about 0.4 g. of substantially pure H - glu - glu - ε - Cbz - lys - ala - ala - OH.

Example 41.

2.8 g. of H - ala - OMe hydrochloride and 6.33 g. of the NHS ester of tBOC-thr are dissolved in 200 ml. of freshly degassed dimethylformamide. The resulting solution is adjusted to pH 8.0 by the addition of diisopropylethylamine and stirred for a period of about four hours while the temperature is maintained at about 25°C. and the pH at 8.0 by the addition of diisopropylethylamine. The reaction mixture is evaporated *in vacuo*, the residual oil is dissolved in methylene chloride, and the methylene-chloride solution is washed twice with 0.2N aqueous sulfuric acid solution saturated with sodium sulfate, once with saturated aqueous sodium chloride solution, twice with saturated aqueous sodium bicarbonate solution, and finally twice with saturated aqueous sodium chloride solution. The washed methylene chloride solution is then dried over anhydrous sodium sulfate and evaporated *in vacuo*, and the residual oil is crystallized from mixtures of ethyl acetate and hexane to give about 4.2 g. of crystalline tBOC - thr - ala - OMe.

Example 42.

3.86 of this tBOC - thr - ala - OMe is dissolved (at a temperature of about 0°C.) in the minimum quantity of trifluoroacetic acid, the solution is stirred at a temperature of about 25°C. for a period of about 45 minutes, and the solution is then evaporated to dryness to give about 4.1 g. of H - thr - ala-OMe trifluoroacetate, in the form of a heavy oil.

Example 43.

4.1 g. of thr - ala - OMe trifluoracetate and 4.1 g. of the NHS ester of tBOC-leu are dissolved in 225 ml. of freshly degassed dimethylformamide. The resulting solution is adjusted to pH 8.0 by the addition of diisopropylethylamine and stirred for a period of about four hours, while the temperature is maintained at about 25 °C. and the pH at 8.0 by the addition of diisopropylethylamine. The reaction mixture is evaporated *in vacuo*, the residual material is dissolved in methylene chloride, and the methylene chloride solution is washed twice with 0.2N aqueous sulfuric acid solution saturated with sodium sulfate, twice with saturated

and finally twice with saturated aqueous sodium chloride solution. The washed methylene chloride solution is then dried over anhydrous sodium sulfate, evaporated *in vacuo*, and the residual solid material is crystallized from ethyl-acetate/hexane to give about 3.4 g. of crystalline tBOC - leu - thr - ala - OMe.

Example 44.

3.1 g. of this tBOC - leu - thr - ala - OMe is dissolved (at a temperature of about 0°C.) in the minimum quantity of trifluoroacetic acid, the solution is stirred at a temperature of about 25°C. for a period of about 45 minutes, and the solution is then added dropwise with vigorous stirring to a large volume (about 100 ml.) of ether. The material which precipitates is recovered by filtration, washed twice with ether, and dried *in vacuo* to give about 2.8 g. of H - leu - thr - ala - OMe trifluoroacetate.

Example 45.

2.8 g. of H - leu - thr - ala - OMe trifluoroacetate and 2.24 g. of the NHS ester of tBOC-met are dissolved in about 60 ml. of freshly degassed dimethylformadide. The resulting solution is adjusted to pH 8.0 by the addition of triethylamine, and stirred for a period of about four hours, while the temperature is maintained at about 25°C. and the pH at 8.0 by the addition of triethylamine. The reaction mixture is evaporated *in vacuo*, the residual material is dissolved in ethyl acetate, and the ethyl acetate solution is washed three times with 0.1N aqueous sulfuric acid solution saturated with sodium sulfate, three times with saturated aqueous sodium bicarbonate solution, and finally with saturated aqueous sodium chloride solution. The washed ethylacetate solution is then dried over anhydrous sodium sulfate and part of the ethyl acetate is evaporated *in vacuo*. The voluminous precipitate which forms is recovered by filtration, washed with a small amount of ethyl acetate, and dried *in vacuo* to give about 1.9 g. of crystalline tBOC-met-leu-thr-ala-OMe.

Example 46.

To 1.5 mg. of tBOC - met - leu - thr-ala - OMe is added 24 ml. of a 1:1 mixture of hydrazine and dimethylformamide. The resulting mixture is stirred for about 5 minutes at room temperature. The resulting solution is evaporated *in vacuo* at a temperature of about 35°C., about 60 ml. of dimethylformamide is added to the residual material, and the resulting solution is evaporated *in vacuo*. 100 ml. of methanol is added, the resulting mixture is evaporated *in vacuo*, and the residual product is crystallized from methanol to give about 1.2 g. of tBOC - met - leu-

Example 47.

253 mg. og tBOC - met - leu - thr - ala hydrazide, prepared as described in Example 46, is dissolved in 15 ml. of freshly degassed dimethylformamide; the solution, which is maintained under a dry nitrogen atmosphere, is cooled to about −40°C., and about 1.3 ml. of 2N anhydrous hydrogen chloride in tetrahydrofuran is added with stirring. About 0.068 ml. of isoamylnitrite is then added, and the resulting mixture is maintained at a temperature between about −20°C. and −15°C. for a period about one hour, at the end of which time the hydrazide has completely reacted to form tBOC - met - leu - thr - ala azide, as may be demonstrated by thin-layer chromatography, utilizing the solvent system chloroform-methanol-water (60:40:10).

Example 48.

To the solution of tBOC - met - leu- thr-ala azide in dimethylformamide, prepared as described in Example 47, is added about 315 mg. of H - glu - glu - ε - Cbz - lys - ala-ala - OH dissolved in 30 ml. of dimethylformamide. The temperature of the solution is then adjusted to −40°C., the pH is adjusted to 8 by the addition of diisopropylethylamine, and the solution is maintained at a temperature between about −20°C. and −15°C., with periodic adjustment of pH to 8.0 by addition of diisopropylethylamine, for a period of about 20 hours, at the end of which time the reaction to form the nonapeptide is substantially complete, as may be shown by thin-layer chromatography on silica gel G using the solvent system ethyl-acetate-pyridine - acetic - acid - water (10:5:1:3). The reaction mixture is evaporated *in vacuo*, the residual material is triturated and washed with water, and the water-washed material is dried to give about 875 mg. of tBOC - met-leu - thr - ala - glu - glu - ε - Cbz - lys - ala-ala - OH.

Example 49.

196 mg. of tBOC - met - leu - thr - ala-glu - glu - ε - Cbz - lys - ala - ala - OH is dried *in vacuo* over phosphorus pentoxide for a period of about 15 hours, thereby removing traces of water, and the resulting dry material is placed in a polyethylene tube containing 2.4 ml. of anisole and 2.4 g. of methionine. The mixture is cooled to a temperature of about −35°C., one ml. of anhydrous hydrogen fluoride is condensed in the tube, and the resulting mixture is stirred at a temperature of about 0°C. for a period of about 45 minutes. At the end of this reaction period, a stream of dry nitrogen is passed through the mixture (still at 0°C.), thereby removing excess of hydrogen fluoride. The residual material is held *in vacuo* at a temperature of about 25°C. for a period of about

acid, and the aqueous acetic acid solution is freeze-dried. The resulting product is dissolved in 45 ml. of water, 5 ml. of mercaptoethanol is added, and the mixture is heated at 45°C. for a period of about 24 hours. The reaction solution is evaporated to dryness *in vacuo*; the residual product is dissolved in 50% aqueous acetic acid; the solution is passed through a gel filtration column and then freeze-dried to give about 75 mg. of H - met - leu - thr - ala - glu - glu - lys-ala - ala - OH.

WHAT WE CLAIM IS:—

1. A process for preparing a hemopeptide, selected from hemopeptides P1, P2, H and S, which comprises coupling together by peptide linkages, utilizing sequential synthesis or block synthesis, the amino acid components in the appropriate sequence as follows: H-val-his - leu - ser - ala - glu - glu - lys - glu-ala - OH for hemopeptide P1; H - val - his-leu - ser - ala - glu - glu - lys - gln - ala - OH for hemopeptide P2; H - val - his - leu - thr-pro - glu - glu - lys - ser - ala - OH for hemopeptide H; and H - met - leu - thr - ala-glu - glu - lys - ala - ala - OH for hemopeptide S; in which process functional groupings, present in such amino acid or peptide components and reactive under the conditions of such coupling, are protected by substituents non-reactive during such coupling and removable without affecting peptide linkages or other protecting substituent retained in subsequent coupling reaction; thereby forming the corresponding protected hemopeptide containing protecting substituents, and subjecting said protected hemopeptide to the action of cleaving agent to remove said substituents, thereby forming the said hemopeptide.

2. A process as claimed in claim 1 as applied to the preparation of hemopeptide P1.

3. A process as claimed in claim 1 as applied to the preparation of hemopeptide P2.

4. A process as claimed in claim 1 as applied to the preparation of hemopeptide H.

5. A process as claimed in claim 1 as applied to the preparation of hemopeptide S.

6. A process for synthesizing hemopeptide P1 from a protected derivative thereof in which functional groupings are protected by substituents removable by vigorous action of a strong acid cleaving agent without substantially affecting peptide linkages, which comprises subjecting the protected hemopeptide P1 derivative to vigorous strong acid cleaving action.

7. A process, as claimed in claim 6, which comprises reacting tBOC - val - his - leu-ser - ala - glu - glu - ε - Cbz - lys - glu-ala - OH with substantially anhydrous hydrogen fluoride thereby forming hemopeptide P1.

8. A process for synthesizing hemopeptide P2 from a protected derivative thereof in which functional groupings are protected by substituents removable by vigorous action of a strong acid cleaving agent without substantially affecting peptide linkages, which comprises subjecting the protected hemopeptide P2 derivative to vigorous strong acid cleaving action.

9. A process, as claimed in claim 8, which comprises reacting tBOC - val - his - leu-ser - ala - glu - glu - ε - Cbz - lys - gln-ala - OH with substantially anhydrous hydrogen fluoride thereby forming hemopeptide P2.

10. A process for synthesizing hemopeptide H from a protected derivative thereof in which functional groupings are protected by substituents removable by vigorous action of a strong acid cleaving agent without substantially affecting peptide linkages, which comprises subjecting the protected hemopeptide H derivative to vigorous strong acid cleaving action.

11. A process as claimed in claim 10, which comprises reacting tBOC - val - his-leu - thr - pro - glu - glu - ε - Cbz - lys-ser - ala - OH with substantially anhydrous hydrogen fluoride thereby forming hemopeptide H.

12. The process for synthesizing hemopeptide S, from a protected derivative thereof in which functional groupings are protected by substituents removable by action of a cleaving agent without substantially affecting peptide linkages, which comprises subjecting the protected hemopeptide S derivative to the action of the cleaving agent.

13. A process as claimed in claim 12, which comprises reacting tBOC - met - leu - thr-ala - glu - glu - ε - Cbz - lys - ala - ala - OH with substantially anhydrous hydrogen fluoride thereby forming hemopeptide S.

14. A process as claimed in claim 2, which comprises reacting ala with glu-NCA to form the dipeptide H - glu - ala - OH, reacting the H - glu - ala - OH with α - tBOC - ε-Cbz - lys - NHS to form the protected tripeptide α - tBOC - ε - Cbz - lys - glu - ala, reacting this tripeptide with trifluoroacetic acid thereby removing the tBOC substituent to form ε - Cbz - lys - glu - ala, reacting the latter compound with glu-NCA to form the protected tetrapeptide H - glu - ε - Cbz-lys - glu - ala - OH and reacting the H - glu-ε - Cbz - lys - glu - ala - OH with glu-NCA to form H - glu - glu - ε - Cbz - lys - glu-ala - OH; reacting H - leu - OMe with his-TCA to form the dipeptide ester his - leu-OMe, reacting this dipeptide ester with tBOC-val-NHS to form the protected tripeptide ester tBOC - val - his - leu - OMe, reacting this protected tripeptide ester with hydrazine and then reacting the resulting hydrazide with nitrous acid to form tBOC - val - his - leu azide; reacting H - ala - OMe with tBOC-ser - NHS to form tBOC - ser - ala - OMe, reacting the latter with trifluoroacetic acid

ser - ala - OMe trifluoroacetate with the said tBOC - val - his - leu azide to produce the protected pentapeptide ester tBOC - val - his-leu - ser - ala - OMe, reacting this protected pentapeptide ester with hydrazine and then reacting the resulting hydrazide with nitrous acid to form tBOC - val - his - leu - ser - ala azide; and reacting this tBOC - val - his-leu - ser - ala azide with the said H - glu-glu - ε - Cbz - lys - glu - ala - OH to form the protected decapeptide, tBOC - val - his-leu - ser - ala - glu - glu - ε - Cbz - lys-glu - ala - OH; and reacting said protected decapeptide with anhydrous hydrogen fluoride to form hemopeptide P1.

15. A process as claimed in claim 3, which comprises reacting ala with gln-NCA to form the dipeptide H - gln - ala - OH, reacting the H - gln - ala - OH with α - tBOC - ε - Cbz-lys - NHS to form the protected tripeptide α - tBOC - ε - Cbz - lys - gln - ala - OH, reacting this tripeptide with trifluoroacetic acid thereby removing the tBOC substituent to form ε - Cbz - lys - gln - ala - OH, reacting the latter compound with glu-NAC to form the protected tetrapeptide H - glu - ε - Cbz-lys - gln - ala - OH, and reacting the H - glu-ε - Cbz - lys - gln - ala - OH with glu-NCA to form H - glu - glu - ε - Cbz - lys - gln-ala - OH; reacting leu-OMe with his-TCA to form the dipeptide ester H - his - leu-OMe, reacting this dipeptide ester with tBOC-val-NHS to form the protected tripeptide ester tBOC - val - his - leu - OMe, reacting this protected tripeptide ester with hydrazine and then reacting the resulting hydrazide with nitrous acid to form tBOC - val - his - leu azide; reacting H - ala - OMe with tBOC-ser-NHS to form tBOC - ser - ala - OMe, reacting the latter with trifluoroacetic acid thereby cleaving the tBOC substituent to form H - ser - ala - OMe trifluoroacetate, reacting the ser - ala - OMe trifluoroacetate with the said tBOC - val - his - leu azide to produce the protected pentapeptide ester tBOC - val-his - leu - ser - ala - OMe, reacting this protected pentapeptide ester with hydrazine and then reacting the resulting hydrazide with nitrous acid to form tBOC - val - his-leu - ser - ala azide; and reacting this tBOC-val - his - leu - ser - ala azide with the said H - glu - glu - ε - Cbz - lys - gln - ala - OH, to form the protected decapeptide, tBOC - val-his - leu - ser - ala - glu - glu - ε - Cbz-lys - gln - ala - OH; and reacting said protected decapeptide with anhydrous hydrogen fluoride to form hemopeptide P2.

16. A process as claimed in claim 4, which comprises reacting H-ala-OMe with tBOC-ser-NHS to form the protected dipeptide tBOC - ser - ala - OMe, reacting this dipeptide with trifluoroacetic acid thereby cleaving the tBOC substituent to form H-ser-ala-OMe, reacting the ser-ala-OMe with α-tBOC - ε-Cbz - lys - NHS to form the protected tripeptide α - tBOC - ε - Cbz - lys - ser - ala - OMe, reacting this tripeptide with aqueous alkali thereby hydrolyzing the methyl ester grouping and reacting the hydrolysis product with trifluoroacetic acid thereby removing the tBOC substituent to form H - ε - Cbz - lys - ser-ala - OH, reacting the latter compound with glu-NCA to form the protected tetrapeptide H - glu - ε - Cbz - lys - ser - ala - OH, and reacting the H - glu - ε - Cbz - lys - ser-ala - OH with glu NCA to form H - glu - glu-ε - Cbz - lys - ser - ala - OH; reacting H-leu - OMe with his-TCA to form the dipeptide ester H - his - leu - OMe, reacting this dipeptide ester with tBOC - val - NHS to form the protected tripeptide ester tBOC-val - his - leu - OMe, reacting this protected tripeptide ester with hydrazine and then reacting the resulting hydrazide with nitrous acid to form tBOC - val - his - leu azide; reacting pro with tBOC - thr - NHS to form tBOC-thr - pro - OH, reacting the latter with trifluoroacetic acid thereby cleaving the tBOC substituent to form H - thr - pro - OH trifluoroacetate, reacting the thr-pro trifluoroacetate with a methylating agent to form H - thr-pro - OMe, reacting this H - thr - pro - OMe with the said tBOC - val - his - leu azide to produce protected pentapeptide ester tBOC-val - his - leu - thr - pro - OMe, reacting this protected pentapeptide ester with hydrazine and then reacting the resulting hydrazide with nitrous acid to form tBOC - val - his-leu - thr - pro azide; and reacting this tBOC-val - his - leu - thr - pro azide with the said H - glu - glu - ε - Cbz - lys - ser - ala-OH to form the protected decapeptide, tBOC-val - his - leu - thr - pro - glu - glu - ε-Cbz - lys - ser - ala - OH; and reacting said protected decapeptide with anhydrous hydrogen fluoride to form hemopeptide H.

17. A process as claimed in claim 5 which comprises reacting ala with ala-TCA to form the dipeptide H - ala - ala - OH, reacting the ala-ala with α - tBOC - ε - Cbz - lys - NHS ester to form the protected tripeptide α-tBOC - ε - Cbz - lys - ala - ala - OH, reacting this tripeptide with trifluoroacetic acid thereby removing the tBOC substituent to form H - ε-Cbz - lys - ala - ala - OH, reacting the latter compound with glu-NCA to form the protected tetrapeptide H - glu - ε - Cbz - lys-ala - ala - OH, and reacting the H - glu - ε-Cbz - lys - ala - ala - OH with glu-NCA to form H - glu - glu - ε - Cbz - lys - ala-ala - OH; reacting ala-OMe with tBOC-thr-NHS ester to form the protected dipeptide ester tBOC - thr - ala - OMe, reacting this protected dipeptide ester with trifluoroacetic acid thereby cleaving the tBOC substituent to form H - thr - ala - OMe trifluoroacetate, reacting the H - thr - ala - OMe trifluoroacetate with tBOC - leu - NHS ester to produce the protected tripeptide ester tBOC-

with trifluoroacetic acid thereby cleaving the tBOC substituent to form leu - thr - ala - OMe, reacting this leu - thr - ala - OMe with tBOC - met - NHS ester thereby forming tBOC - met - leu - thr - ala - OMe, reacting this protected tetrapeptide ester with hydrazine and then reacting the resulting hydrazide with nitrous acid to form tBOC - met - leu-thr - ala azide; and reacting this tBOC-met - leu - thr - ala azide with the said H - glu - glu - ε - Cbz - lys - ala - ala - OH to form the protected nonapeptide, tBOC - met-leu - thr - ala - glu - glu - ε - Cbz - lys-ala - ala - OH; and reacting said protected nonapeptide with anhydrous hydrogen fluoride to form hemopeptide S.

18. Amides, esters and N - acyl analogs of hemopeptide P1 and protected derivatives of hemopeptide P1, its amides, esters and N-acyl analogs.

19. A compound having the structure H-val - his - leu - ser - ala - glu - glu - lys-glu - ala - NH_2.

20. A compound having the structure N-acetyl - val - his - leu - ser - ala - glu - glu-lys - glu - ala - OH.

21. Synthetic hemopeptide P1 when obtained by a process as claimed in claim 6.

22. Synthetic hemopeptide P1 when obtained by a process as claimed in claim 7.

23. Amides, esters and N-acyl analogs of hemopeptide P2 and protected derivatives of hemopeptide P2, its amides, esters and N-acyl analogs.

24. A compound having the structure H-val - his - leu - ser - ala - glu - glu - lys-gln - ala - NH_2.

25. A compound having the structure N-acetyl - val - his - leu - ser - ala - glu-glu - lys - gln - ala - OH.

26. Synthetic hemopeptide P2 when obtained by a process as claimed in claim 8.

27. Synthetic hemopeptide P2 when obtained by a process as claimed in claim 9.

28. Hemopeptide H, its amides, esters and N-acyl derivatives; and protected derivatives thereof.

29. Hemopeptide H.

30. A compound having the structure H-val - his - leu - thr - pro - glu - glu - lys - ser-ala - NH_2.

31. A compound having the structure H-val - his - leu - thr - pro - glu - glu - lys-ser - ala - OH acetate.

32. Synthetic hemopeptide H when obtained by a process as claimed in claim 10.

33. Hemopeptide S, its amides, esters and N-acyl derivatives; and protected derivatives thereof.

34. Hemopeptide S.

35. A compound having the structure H-met - leu - thr - ala - glu - glu - lys - ala-ala - NH_2.

36. A compound having the chemical structure N-acetyl-met-leu-thr-ala-glu-glu-lys-ala-ala - OH.

37. Synthetic hemopeptide S when obtained by a process as claimed in claim 12.

38. Synthetic hemopeptide S, when obtained by a process as claimed in claim 13.

39. Protected derivatives of hemopeptide P1 in which the functional groupings are protected by substituents cleavable therefrom by the vigorous action of a strong acid cleaving agent.

40. A protected derivative as claimed in claim 39 having the structure tBOC - val-his - leu - ser - ala - glu - glu - ε - Cbz - lys-glu - ala - OH.

41. Protected derivatives of the pentapeptide H - glu - glu - lys - glu - ala - OH.

42. Protected derivatives of the pentapeptide H - val - his - leu - ser - ala - OH.

43. Protected derivatives of hemopeptide P2 in which the functional grouping are protected by substituents cleavable therefrom without substantially affecting peptide linkages.

44. A protected derivative as claimed in claim 43, having the structure tBOC - val-his - leu - ser - ala - glu - glu - ε - Cbz-lys - gln - ala - OH.

45. Protected derivatives of the pentapeptide H - glu - glu - lys - gln - ala - OH.

46. A compound having the structure N-formyl - val - his - leu - ser - ala - glu-glu - lys - gln - ala - OH.

47. Protected derivatives of hemopeptide T in which the functional groupings are protected by substituents cleavable therefrom by the vigorous action of a strong acid cleaving agent.

48. A protected derivative as claimed in claim 47, having the structure tBOC - val-his - leu - thr - pro - glu - glu - ε - Cbz-lys - ser - ala.

49. Protected derivatives of the pentapeptide H - glu - glu - lys - ser - ala - OH.

50. Protected derivatives of the pentapeptide val - his - leu - thr - pro.

51. Protected derivatives of hemopeptide H in which the functional groupings are protected by substituents cleavable therefrom by the vigorous action of a strong acid cleaving agent.

52. A protected derivative as claimed in claim 51 having the structure tBOC - met-leu - thr - ala - glu - glu - ε - Cbz - lys-ala - ala.

53. Protected derivatives of the pentapeptide H - glu - glu - lys - ala - ala - OH.

54. Protected derivatives of the tetrapeptide H - met - leu - thr - ala - OH.

55. A process as claimed in claim 1 in which the steps are carried out substantially as hereinbefore described in the foregoing Examples.

56. Hemopeptides and their derivatives when prepared by a process as claimed in any one of claims 1—17 and 55.

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